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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Victor	*Knopov*	*Vancouver, British Columbia*
Kirill	*Dzubanov*	*Vancouver, British Columbia*
Kevin	*Harper*	*Vancouver, British Columbia*

☑ *Additional inventors are being named on the 1 separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Methods and Apparatus for Preparation of Lipid-Encapsulated Therapeutic Agents

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☑ Customer Number 021121 → 021121 PATENT AND TRADEMARK OFFICE

OR

Type Customer Number here

☑ Firm or Individual Name	*Oppedahl & Larson LLP*				
Address	*PO Box 5270*				
Address					
City	*Frisco*	State	*CO*	ZIP	*80443*
Country	*USA*	Telephone	*+1 970 668-2050*	Fax	

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Respectfully submitted,

SIGNATURE *Marina T. Larson*

Date 7/15/99

TYPED or PRINTED NAME *Marina T. Larson, Ph.D.*

REGISTRATION NO. (if appropriate) *32,038*

TELEPHONE *+1 970 668-2050*

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INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Norbert	*Maurer*	*Vancouver, British Columbia*
Kim	*Wong*	*Vancouver, British Columbia*
Pieter	*Cullis*	*Vancouver, British Columbia*

Methods and Apparatus for Preparation of Lipid-Encapsulated Therapeutic Agents

Field of the Invention

This invention relates to a novel method and instruments for making particles of lipid-encapsulated therapeutic agents, and in particular, lipid-encapsulated therapeutic nucleic acid particles which may be useful in antisense therapy or gene therapy. .

Background of the Invention

Large scale manufacturing of fully lipid-encapsulated therapeutic agent particles has not been achieved where there is a significant electrostatic interaction between the lipid and the therapeutic agent. A basic problem is aggregation. Aggregation normally results when charged lipid is mixed with oppositely charged therapeutic agent, resulting in a solution containing a milky flocculent mass which is not useable for further processing, let alone for therapeutic use. The aggregation problem has prevented the development of therapeutic compositions which could be of great utility.

Bench scale formulations using charged lipid and oppositely charged therapeutic agent have been successfully achieved using cationic lipids and anionic nucleic acids in US Pat. No. 5,705,385 to Bally et al. (PCT Applic. No. WO 96/40964; See also US Patent applications S.N. 08/484,282; 08/485,458; 08/660,025; and 09/140,476) and PCT patent Applic. No. WO 98/51278 to Semple et al. (See also US Patent Application S.N. 08/856,374) all assigned to an assignee of the instant invention and incorporated herein by reference. See also Wheeler et al. (1999) Stabilized plasmid-lipid particles: Construction and characterization. Gen. Ther. 6:271-281. These techniques employ an aggregation preventing lipid, such as a PEG-lipid or ATTA-lipid (disclosed in co-pending US Patent Application 08/996,783 which is incorporated herein by reference), which effectively prevents complex aggregate formation. Resulting fully lipid-encapsulated therapeutic agent particles have excellent pharmaceutical characteristics, such as

controlled size (in the 30-250 nm range), full encapsulation (as measured by nuclease resistance, for example) and stability in serum.

WO98/51278 describes a bench scale procedure for the preparation of the lipid-encapsulated therapeutic agent particles which is illustrated in Fig. 1. As shown, this known method employs the two basic steps of lipid hydration and liposome sizing. In the lipid hydration step, a cationic lipid solution (95% EtOH solvent) is added dropwise into an agitated reservoir containing polynucleotide therapeutic agent in citrate buffer (pH 3.8) to a final composition of 40% EtOH, 9.9 mg/ml lipid and 2.0 mg/ml polynucleotide. Lipid particles resulting from this hydration step are typically 400 nm diameter and greater, which is too large for general use as a therapeutic. Because of this, extensive post-formulation processing such as high temperature extrusion (at 65°C) and optionally freeze-thawing (from liquid nitrogen to 65°C waterbath) is required to obtain suitably-sized lipid particles. The efficiency of encapsulation using the method shown in Fig. 1 is fairly high (60-90%) in terms of recovered final drug:lipid ratio, but the absolute efficiency of incorporation of starting polynucleotide into the final particle formulation is sub-optimal (25-45%).

Commercial large scale manufacturing of these particles is not efficiently achieved using traditional methods employed in the liposome field. These problems exist notwithstanding the great deal of art on the manufacturing of liposome/drug formulations that has emerged since the first description of liposome preparation by Bangham, AD. et al. (1965) The action of steroids and streptolysin S on the permeability of phospholipid structures to cations, J. Mol. Biol. 13, 138-147.

Known large scale manufacturing techniques for lipid particles can be broadly classified into the following categories: 1) Lipid Film Hydration (i.e. Passive entrapment); 2) Reverse Phase Evaporation; 3) High-Pressure extrusion; 4) and Solvent injection (dilution) (see for example US Patent Nos. 4752425 and 4737323 to Martin et al). Particular instruments for lipid particle manufacturing disclosed in the art include: US Patent Nos. 5270053 and 5466468 to Schneider et al; Isele, U. et al. (1994) Large-Scale Production of Liposomes Containing

Monomeric Zinc Phthalocyanine by Controlled Dilution of Organic Solvents. J. Pharma. Sci. vol 83(11) 1608-1616; Kriftner, RW. (1992) Liposome Production: The Ethanol Injection Technique, in Bruan-Falco et al., eds, Liposome Derivatives, Berlin, Springer -Verlag, 1992, pp. 91-100; Kremer et al. (1977) Vesicles of Variable Diameter Prepared by a Modified Injection Method. Biochemistry 16(17): 3932-3935; Batzri, S. and Korn, ED. (1973) Single Bilayer Liposomes Prepared Without Sonication, Bioch. Biophys. Acta 298: 1015-1019.

None of the above noted methods or instruments are suitable for scale up of formulations of charged lipid and oppositely charged therapeutic agents with the excellent pharmaceutical characteristics of Bally et al., supra, and Semple et al., supra. The manufacturing techniques set out in Bally et al., supra, and Semple et al., supra were developed only for 1 - 100 ml preparations, and are cumbersome and lead to unsustainable inefficiencies in large scale manufacturing (i.e. at the scale of 20-200 litres).

The instant invention provides, for the first time, methods and instruments for the large-scale preparation of fully encapsulated lipid-therapeutic agent particles where the lipid and therapeutic agent are oppositely charged. These particles are useful as therapeutic compositions and for experimentation and otherwise. It is an object of this invention to provide such methods and instruments.

SUMMARY OF THE INVENTION

In accordance with the present invention, fully lipid-encapsulated therapeutic agent particles are prepared by combining a lipid composition comprising preformed lipid vesicles in a first solvent with a solution of therapeutic agent in a second solvent. The second solvent may be the same as or different from the first solvent, provided that the first and second solvents are miscible with one another and that the resulting mixture is compatible with the maintenance of the vesicle structure. In a preferred embodiment of the invention, effective to provide efficient formation of lipid particles on large scale (for example 20-200 liters), the therapeutic agent solution is introduced into a reservoir containing the preformed lipid vesicles

with gentle stirring. Incubation of this mixture of a period of about 1 hour is sufficient to result in the spontaneous production of fully encapsulated therapeutic agent particles.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a prior art method for preparation of lipid particles including the steps of lipid hydration and liposome sizing;

Fig. 2 shows an embodiment of a method in accordance with the invention for preparation of lipid-encapsulated therapeutic agent particles;

Fig. 3 shows an apparatus in accordance with the invention;

Fig. 4 shows an apparatus in accordance with the invention;

Fig. 5 shows a static mixer which may be used in the apparatus of Fig. 4; and

Fig. 6 shows an extruder which may be used in embodiments of the invention where post-processing including extrusion is desired.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

While the terms used in the application are intended to be interpreted with the ordinary meaning as understood by persons skilled in the art, some terms are expressly defined to avoid any ambiguity. Thus, as used in the specification and claims of this application the term:

charged lipid refers to a lipid species having either a cationic charge or negative charge or which is a zwitterion which is not net neutrally charged, and generally requires reference to the pH of the solution in which the lipid is found.

fully encapsulated refers to lipid particles in which the therapeutic agent is contained in the lumen of a lipid vesicle such as a liposome, or embedded within a bilayer of a lipid particle such that no part of the therapeutic agent is directly accessible to the external medium surrounding the lipid particle. Lipid particles in which the therapeutic agent is fully

encapsulated are distinct from particles in which a therapeutic agent is complexed (for example by ionic interaction) with the exterior of the particle, or from particles in which the therapeutic agent is partially embedded in the lipid and partially exposed to the exterior medium. The degree of encapsulation can be determined using methods which degrade available therapeutic agent. In the case of a polynucleotide, these methods include S1 Nuclease Digestion, Serum Nuclease, and Micrococcal Nuclease analysis. Alternatively, an OliGreen™ assay can be employed. In a quantitative sense, a "fully encapsulated" therapeutic agent is one where less than 10% of the therapeutic agent, and preferably less than 5% of the therapeutic agent in a lipid particle is degraded under conditions where greater than 90% of therapeutic agent is degraded in the free form. It should further be noted that additional therapeutic agent(s) may be associated with the lipid particle by complexation or another manner which is not fully encapsulated with out departing from the present invention.

hydration refers to a common process by which lipid particles, including liposomes, are formed. In this process, the amount of water in the solvent surrounding the lipids is increased from a concentration of around 5% or less (at which concentration the lipid molecules are generally individually solvated) to a concentration of 40-60% or greater (at which lipids spontaneously form into membranes, micelles or particles).

lipid refers to a group of organic compounds that are esters of fatty acids and are characterized by being insoluble in water but soluble in many organic solvents. They are usually divided in at least three classes: (1) "simple lipids" which include fats and oils as well as waxes; (2) "compound lipids" which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids. A wide variety of lipids may be used with the invention, some of which are described below.

preformed vesicle refers to the starting lipid composition used in the method of the invention which contains lipid vesicles. These vesicles have a self-closed structure of generally spherical or oval shape formed from one or more lipid layers and having an interior lumen containing a part of the solvent. The vesicles may be unilamellar, oligolamellar or

multilamellar structures.

The invention disclosed herein relates to a novel method and apparatus for making lipid-encapsulated therapeutic agent particles which is particularly applicable to the large-scale manufacture of such particles when the lipid and therapeutic agent are oppositely charged, such as found in formulations of cationic lipid and anionic polynucleotides. This invention relies upon the surprising and unexpected observation that combining preformed lipid vesicles with a solution of therapeutic agent results spontaneously in the formation of particles of fully lipid-encapsulated therapeutic agent of a therapeutically useful size. Thus, fully lipid-encapsulated therapeutic agent particles are formed in accordance with the invention by a method comprising the step of combining a lipid component comprising preformed lipid vesicles and a solution of the therapeutic agent and incubating the resulting mixture for a period of time to result in the encapsulation of the therapeutic agent in the lipid vesicles. This method has several important characteristics which make it of substantial utility to the art. First, it is a large-scale method which can be used to make substantial quantities (e.g. >100 g) of the encapsulated therapeutic agent. Second, the size of the preformed lipid vesicles is substantially maintained, such that processing of the lipid particles after introduction of the therapeutic agent to obtain particles of therapeutically useful size is not necessary. Third, the efficiency of encapsulation is high. Fourth, the amount of therapeutic agent loaded into the particles is high.

Fig. 2 illustrates one embodiment the method of the invention. Pre-formed empty liposomes in 40% ethanol are placed in a reservoir to which the therapeutic agent, such as a therapeutic oligonucleotide is added. Quite surprisingly, and by a mechanism of membrane inversion yet to be fully understood, a very high level of oligonucleotide is found to be encapsulated within the lipid particle after mixing (drug:lipid ratio = 0.1 to 0.2). Efficiency of encapsulation is also very high, with 60-90% of the starting oligonucleotide being encapsulated in final, patient administration-ready particles. While not intending to be bound by any particular mechanistic theory, it is noted that existing models emphasize the effects of membrane dynamics where oppositely charged particles cause a shift in membrane stresses which cause

inversion of the preformed vesicles, thus encapsulating oligonucleotides that would otherwise be on the outside of the vesicle. Oligonucleotides are not thought to be able to permeate a lipid membrane.

It should be understood that the present method is different from ionic or pH loading of materials into lipid particles using an ionic or pH gradient as described in U.S. Pats. Nos. 5,785,987, 5,380,531 , 5,316,771 and 5,192,549 in several important respects including but not limited to the following. First, in the present invention, the charges on the lipid and the therapeutic agent are opposite to one another. Second, the method of the invention preferably uses a solvent with an organic component such as ethanol. Third, no transmembrane ion-gradient is required. Thus, it is clear that the present invention operates by a different mechanism, albeit one which is not yet fully understood.

The embodiment illustrated in Fig. 2 is an exemplary embodiment of the invention. In a more general sense, fully-encapsulated therapeutic agent particles are formed by combining preformed lipid vesicles with a therapeutic agent solution under conditions of gentle mixing and then incubated for a period of time sufficient to result in formation of the desired product. The preformed lipid vesicles may be added to a reservoir containing the therapeutic agent solution, provided care is taken to avoid disruption of the lipid particles. Thus, the term "combining" does not define the specifics of mixing of the therapeutic agent solution and the preformed lipid vesicles.

The preformed lipid vesicles used in the method of the invention may suitably be empty vesicles. In some cases, however, it may be desirable to associate an additional therapeutic agent (for example a neutral therapeutic agent) with the preformed lipid vesicles prior to combination with the charged therapeutic agent solution.

The preformed lipid vesicles are prepared in a solution of ethanol or other organic solvent using a simple lipid hydration step. The percentage of ethanol or other organic solvent must be selected such that the lipid particles do not disassemble or redissolve into the solvent (generally at >60% ethanol) but provide conditions which permit the spontaneous membrane

inversion encapsulation process of the invention (approx. 5%-50% ethanol). Preferably, lipid particles are prepared by a method which does not require a sizing step however, sizing steps may be employed to generate the empty liposomes. Sizing methods such as high temperature/ high pressure extrusion, freeze-thawing, sonication, etc. may be employed, because the process will not involve the therapeutic agent, and hence will not damage the agent.

The preformed lipid vesicles may be formed in accordance with the invention using an apparatus of the type shown in Figs. 3 and 4. Using these apparatus, lipid solution is introduced into a stirred reservoir containing buffer through a dispensing head having one or more ports with an orifice diameter of 2 mm or less to produce the preformed vesicles. Little or no sizing after formation is required when this apparatus is used.

The size range of preformed lipid vesicles influences the size of the final particle, so a preferred preformed lipid particle is 80-150 nm, preferably 90-100 nm. Techniques found to be useful for making the preformed lipid vesicles include the use of a static mixer, dispersion of ethanolic lipid in a reservoir of aqueous buffer, or classical lipid film hydration. Any method may be employed, but the method will effect the size of the empty liposome, approximately as follows:

Method	Median Empty Liposome Size
continuous addition (process of example 1)	170-200 nm
Static Mixer (Low Turbulence)	150-170 nm
Static Mixer (High Turbulence)	100-120 nm

The method of the invention employs relatively dilute solutions of lipid particles and therapeutic agent. In general, the therapeutic agent solution will have a concentration of 1 to 1000 mg/ml, preferably 10-50 mg/ml of the therapeutic agent, to yield a final concentration (after mixing with the preformed lipid vesicles) in the range of 0.2 – 0.4 mg/ml, preferably about 0.25 mg/ml. Preformed lipid vesicles are combined with the therapeutic agent solution such that the

resulting lipid concentration (after mixing with therapeutic agent solution) is about 1.5 – 3.0 mg/ml (about 2-4 mM), preferably 2.5 mg/ml (3.3 mM). A preferred composition for preformed vesicles for use with polynucleotide therapeutic agent is made at the standard lipid ratios (PEG-cerC14: DODAP: DSPC:Chol (molar ratios 5:25:25:45). This solution, in 100% ethanol, is diluted to 5-50% ethanol, preferably 40% ethanol by mixing with aqueous buffer,, for example 300 mM citrate, pH 4.0.

In one embodiment of the invention, the therapeutic agent is introduced into a reservoir containing the preformed liposomes through a dispensing head one or more ports using an apparatus of the type depicted in Fig. 3. Oligonucleotide is provided in aqueous ethanol (medical grade) at pH 6.0-6.5. The concentration of ethanol may be the same as the ethanol concentration of the lipid solution (in which case no change takes place on mixing), or it may be greater or less than the concentration of the lipid solution provided that the therapeutic agent is soluble in the solution and the desired final concentrations can be obtained . A suitable solvent for oligonucleotides is 40% ethanol.

Encapsulation results upon gently stirring the lipid solution and the oligonucleotide solution together until well mixed, then incubating with no mixing or gentle mixing for 1 hour at 40°C. During hydration, concentration of lipid is about 2.5 to 10 mg/ml, oligonucleotide is about 0.25 to 1.25 mg/ml and EtOH is about 40%. Resulting drug:lipid ratio by weight of particles is 0.1 to 0.2. Median particle sizes of 90-100 nm diameter are obtained. If non-optimal concentrations of lipid and oligonucleotide are used, extrusion may be required to further reduce lipid particle size to the desired 90-100 nm size. Other post formulation techniques are employed as usual. This highly efficient method encapsulates 60-90% of initial oligonucleotide and is suitable for the 20 – 200 litre range.

Preparation and Selection of Lipids and Therapeutic Agents

The lipid particles used in the present invention are formed from a combination of several types of lipids, including at least (1) a charged lipid, having a net charge which is opposite to the charge of the therapeutic agent; and (2) a modified lipid including a modification such as a polyethylene glycol substituent effective to limit aggregation. In addition, the formulation may contain a neutral lipid or sterol. In formulating the lipid particles using all of the above-mentioned components, the following amounts of each lipid components are suitably used: 10 to 40 mol % charged lipid; 25 to 45 mol% neutral lipid, 35-55 mol% sterol; and 0.5 to 15 mol % modified lipid. Specific lipid components may be selected from among the following non-limiting examples.

Charged Lipids

A wide variety of charged lipids and oppositely charged therapeutic agents may be used with the invention. Examples of such compounds are available and known to persons skilled in the art. The following lists are intended to provide illustrative, non-limiting examples.

Cationic charged lipids at physiological pH include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol") and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, Lipofectin™ (commercially available cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE"), from GIBCO/BRL, Grand Island, New York, USA); Lipofectamine™ (commercially available cationic liposomes comprising N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA") and DOPE from GIBCO/BRL); and

Transfectam™ (commercially available cationic lipids comprising dioctadecylamidoglycyl carboxyspermine ("DOGS") in ethanol from Promega Corp., Madison, Wisconsin, USA).

Some cationic charged lipids are titrateable, that is to say they have a pKa at or near physiological pH, with the significant consequence for this invention that they are strongly cationic in mild acid conditions and weakly (or not) cationic at physiological pH. Such cationic charged lipids include, but are not limited to, N-(2,3-dioleyloxy)propyl)-N,N-dimethylammonium chloride ("DODMA") and 1,2-Dioleoyl-3-dimethylammonium-propane ("DODAP").

Anionic charged lipids at physiological pH include, but are not limited to, phosphatidyl inositol, phosphatidyl serine, phosphatidyl glycerol, phosphatidic acid, diphosphatidyl glycerol, poly(ethylene glycol)-phosphatidyl ethanolamine, dimyristoylphosphatidyl glycerol, dioleoylphosphatidyl glycerol, dilauryloylphosphatidyl glycerol, dipalmitoylphosphatidyl glycerol, distearyloylphosphatidyl glycerol, dimyristoyl phosphatic acid, dipalmitoyl phosphatic acid, dimyristoyl phosphatidyl-serine, dipalmitoyl phosphatidyl-serine, brain phosphatidyl serine, and the like.

Some anionic charged lipids may be titrateable, that is to say they would have a pKa at or near physiological pH, with the significant consequence for this invention that they are strongly anionic in mild base conditions and weakly (or not) anionic at physiological pH. Such anionic charged lipids can be identified by one skilled in the art based on the principles disclosed herein.

Neutral Lipids and sterols

The term "neutral lipid" refers to any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form a physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides and diacylglycerols.

Modified Lipids

Certain preferred formulations used in the invention include aggregation preventing lipids such as PEG-lipids or polyamide oligomer-lipids (such as an ATTA-lipid), and other steric-barrier or "stealth"-lipids. Such lipids are described in US Patent Nos. 4320121 to Sears, 5,820,873 to Choi et al., 5,885,613 to Holland et al., WO 98/51278 (inventors Semple et al.), and US Patent Application Serial No. 09/218988 relating to polyamide oligomers, all incorporated herein by reference. These lipids prevent precipitation and aggregation of formulations containing oppositely charged lipids and therapeutic agents. These lipids may also be employed to improve circulation lifetime *in vivo* (see Klibanov et al. (1990) FEBS Letters, 268 (1): 235-237), or they may be selected to rapidly exchange out of the formulation *in vivo* (see US Pat. No. 5885613). Particularly useful exchangeable lipids are PEG-ceramides having shorter acyl chains (i.e, C14 or C18, referred to herein as PEG-CerC14 and PEG-CerC18) or PEG-PE having a C14 acyl chain.

Some lipid particle formulations may employ targeting moieties designed to encourage localization of liposomes at certain target cells or target tissues. Targeting moieties may be linked to the outer bilayer of the lipid particle during formulation or post-formulation. These methods are well known in the art. In addition, some lipid particle formulations may employ fusogenic polymers such as PEAA, hemagluttinin, other lipo-peptides (see US Patent applications SN 08/835,281, and 60/083,294, all incorporated herein by reference) and other features useful for *in vivo* and/or intracellular delivery.

Sometimes the lipids particles and therapeutic agents of the invention are provided in organic solvents, detergent solutions, or the like, in order to provide solution streams suitable for mixing compounds of different charge and different solubility in water. Preferred organic solvents and detergent solutions are set out below.

For purpose of this specification, "organic solvent" means either a completely organic solvent (i.e. 100% ethanol) or a partially organic solvent (such as ethanol in water, ie. 20% ethanol, 40% ethanol, etc.). A wide variety of water miscible organic solvents may be used

including ethanol or other alcohols, acetonitrile, dimethylformamide, DMSO, acetone, other ketones, and the like. Solvents with greater or lesser polarity may be useful in some cases.

Detergent solutions include β-D-glucopyranoside, Tween 20 and those set out in WO 96/40964 and US Patent application SN 09/169573, both incorporated herein by reference, and any other detergent or steric barrier compound that can provide the same solubility features, and/or can prevent particle aggregation during mixing of oppositely charged lipid and therapeutic agent.

Preferably all organic solvents or detergent solutions are pharmaceutically acceptable in trace amounts, or greater, in order that the formulation process does not preclude patient administration.

Therapeutic Agent

The methods of the invention employ oppositely charged lipid and therapeutic agent in order to spontaneously load the therapeutic agent into the lipid particle. Once loading is complete, pH of the particle solution may be changed, possibly neutralizing one or other of the components. The description of "anionic" or "cationic" refers to the net charge on the compound at the pH when the particle is being formed.

Anionic therapeutic agents include any therapeutic agent with a net negative charge, or having a negatively charged group that is able to interact with a cationic lipid without being blocked by other cationic charge groups of the therapeutic agent. Such therapeutic agents include any known or potential therapeutic agent, including all drugs and compounds such as, but not limited to, oligonucleotides, nucleic acids, modified nucleic acids (including protein-nucleic acids and the like), proteins and peptides with negative charge groups, conventional drugs such as plant alkaloids and analogues having negative charge groups, and the like. Therapeutic agents which are not inherently anionic may be derivatized with anionic groups to facilitate their use in the invention.

Cationic therapeutic agents include any therapeutic agent with a net positive charge, or having a positively charged group that is able to interact with a negative lipid without being blocked by other negative charge groups of the therapeutic agent. Such therapeutic agents include any known or potential therapeutic agent, including all drugs and compounds such as, but not limited to modified nucleic acids linked to cationic charges, proteins and peptides with positive charge groups, conventional drugs such as plant alkaloids and analogues having positive charge groups, and the like. Therapeutic agents which are not inherently cationic may be derivatized with cationic groups to facilitate their use in the invention. For example, paclitaxel can be derivatized with a polyglutamic acid group linked to the 2' carbon.

Typically, charged therapeutic agents are initially provided in buffered aqueous solution, generally containing some amount of ethanol or other organic solvent. Salt concentration can strongly effect the self assembly process (see US Patent application SN 09/169573 incorporated herein by reference) employed in the invention, so the buffered salts employed need to be carefully selected. Further, all buffers must be pharmaceutically acceptable, as traces may remain in the final formulation. A preferred buffer is 300 mM citrate buffer. The amount of ethanol or other organic solvent which may be included is controlled by the solubility of the therapeutic agent in the aqueous organic mixture, and also by the desired characteristics of the final mixture of therapeutic agent and preformed lipid vesicles.

The selection of lipids and therapeutic agents are made to work in concert to provide fully lipid-encapsulated compositions. Thus, if the therapeutic agent is a polyanionic oligonucleotide, the lipid components should be selected to include lipids which are cationic under the conditions at which the lipid particles are formed. Conversely, if the therapeutic agent is cationic, the lipids components should be selected to include lipids which are anionic under the conditions at which the lipid particles are formed. This does not mean that all of the lipids included in the lipid solution must be charged, nor does it exclude the incorporation of some quantity of like-charged lipids or of zwiterrionic lipids. It merely means that the lipid solution

should include lipids which have a net charge which is opposite to the net charge of the therapeutic agent.

In accordance with the invention, the lipid components are provided as preformed vesicles in a first solvent, and the therapeutic agent is provided in a second solvent, which may be the same or different from the first solvent. When different from one another, the two solvents are selected such that they are miscible in the concentration range achieved when practicing the invention, and such that the lipid vesicles will remain intact in the final mixture. This strategy requires careful selection of solutions such that they will both solubilize the lipid and therapeutic agent (which may have quite different solubilities and hydrophobic characteristics) and yet will permit mixing in a single phase. Two useful methods are to provide 1) lipid in aqueous detergent solution and therapeutic agent in aqueous buffer; or 2) lipid in organic solvent, such as ethanol, and therapeutic agent in aqueous buffer.

Optional Sizing of Lipid Particles

At the end of the incubation step, the method of the invention results in spontaneous-formed fully-encapsulated therapeutic agents particles having a size which is acceptable for therapeutic use and which can be predicted based on the starting side of the preformed lipid vesicles. Thus, in general, a sizing step of the type known in the art is not necessary. Should further sizing of the product particles be desired, however, an optional step for sizing of the resulting lipid particles may be employed. Further, a sizing step may be employed as part of the preparation of the preformed vesicles prior to the introduction of the therapeutic agent. There are several methods for the sizing of lipid particles, and any of these methods may generally be employed when sizing is used as part of the invention.

The extrusion method is a preferred method of liposome sizing. *see* Hope, MJ et al. Reduction of Liposome Size and Preparation of Unilamellar Vesicles by Extrusion Techniques. In: Liposome Technology (G. Gregoriadis, Ed.) Vol. 1. p 123 (1993). The method consists of extruding liposomes through a small-pore polycarbonate membrane or an asymmetric

ceramic membrane to reduce liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller pore membranes to achieve gradual reduction in liposome size.

A variety of alternative methods known in the art are available for reducing the size of a population of liposomes ("sizing liposomes"). One sizing method is described in U.S. Patent No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in diameter. Homogenization is another method; it relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. The size of the liposomal vesicles may be determined by quasi-electric light scattering (QELS) as described in Bloomfield, Ann. Rev. Biophys. Bioeng., 10:421-450 (1981), incorporated herein by reference. Average liposome diameter may be reduced by sonication of formed liposomes. Intermittent sonication cycles may be alternated with QELS assessment to guide efficient liposome synthesis.

Preferred sizes for liposomes made by the various liposome sizing methods will depend to some extent on the application for which the liposome is being made, but will in general fall within the range of 25 to 250 nm. Specific examples of suitable sizes are set out in the Examples below.

Apparatus for Making Fully Lipid-Encapsulated Therapeutic Agent Particles

The method described above and depicted in Fig. 2 may be put into practice using the apparatus described and set forth below. Addition of a therapeutic agent solution (for example an oligonucleotide solution in ethanol/buffer) to a reservoir of preformed lipid vesicles may be achieved using the apparatus shown in Fig. 3. Stainless-steel reaction vessel **20** (volume

20 – 200 litres), maintained at 28 °C by thermal jacket **21**, is approximately half filled with the solution of preformed lipid vesicles from tank **22** via a pump **25** and through loading port **23**. Agitator **13**, driven by sanitary mixer **3**, is engaged to stir very gently. Optionally, reaction vessel **20** may include interior baffles to increase mixing efficiency and minimize foaming. The desired therapeutic agent solution flows from tank **1** into reaction vessel **20** via a pump **2**. The therapeutic agent may be injected under the surface of the agitated therapeutic agent solution, or it may be dropped onto the surface of the agitated solution.

In one embodiment of the apparatus, which can also be used for the initial preparation of preformed vesicles, the therapeutic agent solution is introduced through a spraying coil **14**. Spraying coil **14** is a hollow stainless steel coil having internal diameter 10 mm, and curved at 60-70% of the radius of the tank, being at least 2 inches from the internal wall surface. At least one, and preferably a plurality (for example twenty or more) ports or apertures of diameter 2mm or less provide a stream of therapeutic agent solutions into the vessel. Port diameters of 1mm or 0.25 are preferred, and smaller port sizes are useable. Multiple spraying coils may be employed, if desired.

The therapeutic agent solution is added at a steady flow rate of approximately 200-300 ml/min. Once the therapeutic agent solution is added to the desired amount to reaction vessel **20**, agitator **13** is disengaged and the solution is incubated according to the protocol. Prepared solution is transferred to lower reservoir **11** to await further processing. Defoaming elbows **12** (10 mm diameter, stainless steel) are used to deliver suspensions onto the interior wall surface of the vessel **20** to avoid foaming. All parts of the instrument are custom made from stainless steel, solvent resistant plastic, or any other pharmaceutically acceptable materials.

The apparatus of Fig. 3 may also be used in the reverse configuration, i.e., by adding preformed lipid vesicles to a reservoir containing therapeutic agent solution. In either configuration, the preformed lipid vesicles should be handled with care to avoid disruption of the vesicles.

If a post-loading extrusion step is desired, the prepared solution may be cycled through the optional continuous flow extrusion circuit **24**. Circuit **24** includes diaphragm-metering pump (Bran & Luebbe, Model: N-D31) **10**, a heat exchange system **5**, which raises the solution temperature to a temperature suitable for extrusion, generally 65°C, and extruder **4**. Extruder **4** is a custom designed continuous flow extruder set out in Fig. 6. This extruder includes two plates of 25 mm thickness, 316 SS and a secure closing system which allows use of pressures up to 1000 psi. The plates form an internal volume to 125 ml and surface area sufficient for a 142 mm membrane. Membranes are polycarbonate membranes having 142 mm diameter and preferably either 50nm, 80 nm or 100 nm pore size (Poretics, Inc. or Nucleopore, Inc.) though other sizes may be used. Two stacked membranes are used for each pass.

It is convenient to use two reaction vessels, **11** and **20** to collect extruded material during these extrusion steps. The extruded material is transferred to lower reservoir **11** prior to each cycle to ensure the exact amount of passes for each extrusion circuit. Flow rate through the extrusion circuit is from 50 to 2000 ml/min.

As indicated, the extrusion step is optional, based on the desired size of the lipid-therapeutic agent particles. After this decision is made, and extrusion done if required, the particles are then ready for final processing. The suspension of particles is dialyzed, such as by tangential flow dialysis or diafiltration, to replace buffer and remove unwanted components. The replacement buffer is a traditional pharmaceutically acceptable buffer such as Phosphate Buffer Solution (PBS) (pH 7.4). Unwanted components include ethanol, unencapsulated oligonucleotide or initial buffer. Again from Fig. 3, vessel **16** is a diafiltration vessel, which may be in parallel to increase flow rate. Typically, diafiltration systems include hollow fiber cartridge(s) **16** (UFP-100-C-55 (100,000 mw cut-off; 3.2m^2), A/G Technology Corp.), which may be used in parallel to increase hollow fiber surface area and consequently permeate flow rate. The circuit also includes sanitary rotary lobe pump **19** (Lobtop 350, Teknoflow, Inc.), process vessel **15** (Polysulfone, vol. 5L, A/G Technology Corp), buffer tank **8** (vol. 50 L, polypropylene, Nalgene) and 316 stainless steel flexible tubing (Inland Machinery). The

suspension is circulated through the ultrafiltration column under low pressure (10 psi), and permeate is driven out. Ethanol and free oligonucleotide with approx 8,000 MW should be fully removed in permeate. Replacement buffer for diafiltration, such as PBS, flows from tank **8** according to the vacuum created by the diafiltration process. Permeate may optionally be collected for recovery of unencapsulated therapeutic agent, or other components of the system. Typical permeate flow rate during diafiltration , for one cartridge ($3.2m^2$) is 1.0-1.4 L/min. Temperature during diafiltration is 26-28 °C. A de-foaming circuit, including vessel **17** may also be employed in the diafiltration circuit.

Once the buffer is replaced and unwanted components are removed, the suspension is ready for final concentration. This is achieved by continuing the circuit without the addition of replacement buffer. This system routinely concentrates a 1 mg/ml (oligonucleotide) solution to 3 mg/ml for packaging and distribution. Final concentrated product is collected and stored in pharmaceutically acceptable plastic, glass or stainless steel to await sterile filtration and packaging according to methods known in the art.

Fig. 4 shows an alternative apparatus which can be used for preparation of preformed lipid vesicles for use in the invention using a continuous flow hydration (mixing) procedure with a static mixer **5** (or motionless mixer). If the optional extrusion circuit is not required, the static mixer design can be further simplified by elimination of the lower reservoir **11.** The diafiltration tanks **21**, defoaming tank **19**, and storage loops can be attached directly to the first tank **20.**

A suitable static mixer **5** is the Statiflo Motionless Mixer (Statiflo Inc. Toronto). The static mixer design is set out in Fig. 5. The static mixer relies on the principles of radial momentum transfer, flow division and shear plane reversal. These transport phenomena combine to eliminate concentration, velocity and thermal gradients. By using an elliptical shape of mixing elements, smooth transitions are possible and no energy is wasted in back mixing. Thus, static mixer will completely blend and disperse two fluids in short lengths of piping. The mixing elements used are made in two patterns: a left-handed inclined ellipse (LH) provides clockwise

rotational flow and the right-handed inclined ellipse (RH) provides counterclockwise rotational flow. The elements are connected at 90° angels to each other and thc two element patterns are alternated in the following series: RH, LH, RH, LH, etc. In some designs, mixing elements create regions of relatively greater turbulence alternating with relaxation zones.

Because a static mixer operates in a pipeline, fluids proceed axially through the line in a flow regime defined by the degree of turbulence characterized by the dimensionless Reynolds number N_{re}. $N_{re} < 500$ is laminar flow; $500 < N_{re} < 2000$ is transitional; $N_{re} > 2000$ is fully turbulent. N_{re} dictates the flow regime and therefore determines how many mixing elements are necessary for a particular application. Approximately 4 – 6 mixing elements are required for satisfactory mixing in the examples hereto, when using lipid and therapeutic agents in the concentrations, volumes, mixing ratios, port sizes and flow rates employed,

The main benefit of a static mixer is that when ethanolic lipid and buffer solution concentrations are constant, and flow rates are precisely metered, resulting particle sizes and characteristics can be prccisely defined. Other benefits of static mixer in the invention include the following: Mixing conditions are constant throughout the procedure and concentration of the ethanol in the reaction cell is fixed over time; Batch size is unlimited since receiving tanks can be systematically filled; easy installation, operation and cleaning; absence of moving parts; cost effectiveness; limited energy loss, back mixing and redundant mixing; Adjustability of N_{re} and shear stress; long service life; reduced manpower requirements; and no external power source required.

Examples

Materials used in the following examples are supplied as follows: Distearoylphosphatidylcholine (DSPC), was purchased from Northern Lipids (Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1) was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased from Avanti Polar Lipids. Cholesterol (CHOL) was purchased from Sigma Chemical Company (St. Louis, Missouri, USA).

PEG-ceramides were synthesized by Dr. Zhao Wang at Inex Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Massachusetts, USA). All lipids were > 99% pure. Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all purchased from commercial suppliers. Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL (PEG-Ceramides were prepared at 50 mg/mL). Antisense oligonucleotide INX-6295 is a 16mer phosphorothioate oligodeoxynucleotide (optionally tritiated for distribution studies) which is hybridizable with the initiation codon region of human c-myc mRNA, having sequence:

5' T AAC GTT GAG GGG CAT 3' (SEQ ID. No 1)

INX-6295 is synthesized using standard oligonucleotide amidite synthesis methods (Inex Pharmaceuticals USA, Inc., Hayward, CA).

Analytical Methods: Assays employed to determine if a lipid-therapeutic agent is "encapsulated" such as being "fully encapsulated" are set out in WO 98/51278, and incorporated herein by reference. Such methods include S1 Nuclease Digestion, Serum Nuclease, and Micrococcal Nuclease analysis.

The Oligreen Assay was used to quantify the amount of oligonucleotide loaded into the vesicles. A fluorescent dye binding assay for quantifying single stranded oligonucleotide in aqueous solutions was established using a Biolumin™ 960 fluorescent plate reader (Molecular Dynamics, Sunnyvale, California, USA). Briefly, aliquots of encapsulated oligonucleotide were diluted in HEPES buffered saline (HBS; 20mM HEPES, 145mM NaCl, pH 7.5). A 10 µL aliquot of the diluted sample was added to 100 µL of a 1:200 dilution of Oligreen™ reagent, both with and without 0.1% of Triton X-100 detergent. An oligo standard curve was prepared with and without 0.1% Triton X-100 for quantification of encapsulated oligo. Fluorescence of the Oligreen™-antisense complex was measured using excitation and emission wavelengths of 485nm and 520nm, respectively. Surface associated antisense was determined by comparing the fluorescence measurements in the absence and presence of detergent.

Example 1

Empty preformed vesicles were prepared from a lipid mixture containing PEG-CerC14, DODAP, DPSC and CHOL in a molar ratio of 5:25:25:45. The four lipids were dissolved in a 100% ethanol to a total lipid concentration of 25 mg/ml (33 mM). The ethanolic lipid was then introduced through an injection port with an orifice diameter of 0.25 mm using the apparatus of Fig. 3 into a reservoir containing 300 mM citrate buffer, pH 4.0. The reservoir and all solutions were at room temperature. The total volume of ethanolic lipid was 6 liters, and the flow rate for lipid introduction was 200-300 ml/min. The total volume of citrate buffer was 9 liters. The resulting 15 liter mixture had an ethanol concentration of 40% and 180 mM citrate. Vesicles of 170 ± 20 nm median diameter were generated. The empty preformed vesicles were sized to 90-120 nm median diameter by 1-3 passes through the extrusion circuit (65°C) at low pressure (100 p.s.i., reduced from classical 500-1000 p.s.i.) using two stacked 80 nm membranes. The empty preformed vesicles were then pooled in reservoir 20 of the apparatus of Fig. 3 and maintained at 40°C until addition of therapeutic agent solution.

Example 2

Fully lipid-encapsulated oligonucleotide particles were made using the embodiment of the invention illustrated in the Fig. 2 to take advantage of the remarkable finding that preformed empty liposome vesicles, will spontaneously encapsulate oligonucleotides when mixed with the buffered oligonucleotide solution.

Preformed vesicles of example 1 were used to make fully lipid-encapsulated therapeutic agent particles using oligonucleotide INX-6295 (Seq. ID No. 1) as the therapeutic agent. Oligonucleotide INX-6295 in distilled water was diluted by the addition of 100 % ethanol to form a various solutions of 10, 20, 30 40 or 50 mg/ml oligonucleotide in 40% ethanol. The ethanolic oligonucleotide was added to the preformed vesicles in reservoir 20 at 40°C with gentle mixing. The amount and volume of ethanolic oligonucleotide was calculated to provide a

final drug:lipid ratio of 0.1 to 0.25 by weight. The mixture was then incubated at 40°C with gentle and periodic mixing for 1 hour. After incubation, the solution was processed by diafiltration to strip free or excess associated oligonucleotide, remove ethanol and exchange the buffer system to phosphate buffered saline (PBS), pH 7.4. Concentration, sterile filtration and packaging complete the preparation of a commercial product.

Example 3

The procedure of Example 2 was repeated with changes to various parameters to determine which might be critical to the preparation of fully lipid-encapsulated therapeutic agent particles in accordance with the invention. In these experiments, the total oligonucleotide recovery (yield), the total lipid recovery (yield) and the encapsulation efficiency were considered as indications of the quality of the product and the process.

Total oligonucleotide recovery was calculated using the formula :

$$\frac{\text{final oligo concentration (mg/ml) X final volume (ml)}}{\text{initial oligo concentration (mg/ml) X initial volume (ml)}} X\ 100\%$$

Total lipid recovery was calculated using the formula :

$$\frac{\text{final lipid concentration (mg/ml) X final volume (ml)}}{\text{initial lipid concentration (mg/ml) X initial volume (ml)}} X\ 100\%$$

Encapsulation Efficiency (E.E.) was calculated using the formula:

$$\frac{\text{initial oligo (mg/ml) / initial lipid (mg/ml)}}{\text{final oligo (mg/ml) / final lipid (mg/ml)}} X\ 100\%$$

The percentage of oligo that is encapsulated (i.e., incorporated in bilayers or entrapped in the interior of the lipid particle) was determined with the OliGreen assay described above.

To assess the significance of the initial drug to lipid ratio, the experiment was conducted with two different starting ratios. The results are summarized in Table 1. No change in the size of the vesicles was observed in the process of loading the oligonucleotide.

Table 1

Initial Drug/Lipid Ratio	Oligo Yield %	Lipid Yield %	Encap Oligo %	Vesicle size (nm)	Final Drug/Lipid Ratio	E.E. %
0.1	80-90	70-80	≥90	106	0.1	100
0.2	60-78	70-75	≥80	119	0.17-0.2	85-100

To assess the significance of incubation temperature, the experiment was conducted at room temperatures and at two elevated temperatures for 1 hour. The results are summarized in Table 2. As shown, the higher temperature of 60°C begins to impair the efficiency of the process, and to lead to an increase in particle size. Thus, lower temperatures are preferred.

Table 2

Incubation Temp (°C)	Oligo Yield %	Encaps Oligo %	Vesicle Size (nm)
RT (20-22)	73	90	114
40	84	91	109
60	52	83	140

To assess the significance of incubation time,. the experiment was conducted at three incubation times and an incubation temperature of 40 °C. The results are summarized in Table 3. As shown, the yield improves between 0.5 hours and 1 hour, but increased incubation

time beyond an hour does not result in a substantial improvement. Thus, the most efficient process in the apparatus used will employ an incubation time of about 1 hour.

Table 3		
Incubation time (hr)	Oligo Yield %	Encapsulated Oligo %
0.5	22	92
1.0	60	94
2.0	56	95

To assess the significance of buffer concentration in the oligonucleotide solution, incubation time, the experiment was conducted at four different concentrations of citrate buffer and an initial drug/lipid ratio of 0.1. The results are summarized in Table 4.

Table 4			
Citrate Buffer Conc (mM)	Oligo Yield %	Encapsulated Oligo %	Vesicle Size (nm)
50	100	94	80
100	88	90	90
200	89	91	93
300	80-90	92	106

To assess the significance of the initial ethanol concentration during the mixing step, the experiment was conducted with 3 different initial ethanol concentrations at each of two initial drug to lipid ratios. The results are summarized in Table 5. There appears to be an optimum ethanol concentration which is different for each starting oligo/lipid ratio. In an addition experiment not reported in the Table, an initial ethanol concentration of 50 % was used

with an oligo/lipid ratio of 0.1. Significant problems of unknown cause were encountered in this experiment and no yield of product was obtained.

Table 5

Initial EtOH %	Initial Drug/Lipid	Oligo Yield %	Encaps Oligo %	Vesicle size (nm)	Final Drug/Lipid	E.E. %
33	0.2	42-47	88	115	0.12	60
40	0.2	70	82	114	0.15	75
43	0.2	64	62	105	0.19	95
36	0.1	52-66	85-89	110	nd	nd
43	0.1	90-100	84-89	116	nd	nd
45	0.1	90-100	90-92	108	nd	nd

To assess the significance of initial oligonucleotide concentration (and thus of the volume of therapeutic agent solution to obtain the same initial drug to lipid ratio), stock solutions at four different concentrations of oligonucleotide were used. The results are summarized in Table 6. As shown, this parameter does not appear to be critical to the results obtained using the method of the invention.

Table 6

Oligo Stock mg/ml	Initial Drug/Lipid	Oligo Yield %	Encaps Oligo %	Vesicle Size (nm)
10	0.1	85	90	106
20	0.1	80	88	112
30	0.1	87	90	110
40-50	0.1	80-90	88-94	106

Example 4

To demonstrate the applicability of the invention to larger therapeutic agents, plasmid pINEX L1018, a 5.5 kb plasmid encoding the luciferase gene linked to a CMV promoter, and also carrying SV40 enhancer elements and an AmpL gene was loaded into preformed lipid vesicles.

Preformed lipid vesicles were prepared by slowly adding 10 mg of lipids (DSPC/CHOL/.DODAP/PEG-CerC14 in a 20/45/25/10 mol % ratio) dissolved in 100% ethanol to 25 mM citrate buffer (25 mM citric acid, 255 mM sucrose, adjusted to pH 4 with sodium hydroxide). Both solutions were prewarmed to 40°C before mixing. The final ethanol concentration was 40% (v/v). The ethanolic dispersion of lipid vesicles was extruded 2X through 2 stacked 100 nm polycarbonate filters at room temperature. 0.25 mg of plasmid DNA in 40% ethanol was added to the lipid vesicles at room temperature, followed by a 1 hour incubation of the sample at 40°C. The initial plasmid/lipid ratio was 0.025. Subsequently, the sample was dialyzed against 2L of 25 mM saline, pH 7.5 (20 mM HEPES, 150 mM NaCl) for a total of 18-20 hours.

Trapping efficiency was determined after removing remaining external plasmid DNA by anion exchange chromatography on a DEAE Sepharose CL6B column. Plasmid DNA was quantified using the DNA Binding System PicoGreen lipid by inorgranic phosphate assay according to Fiske and Subbarrow after separation from the plasmid by a Bligh Dyer extraction. In addition, the final lipid concentration was determined by incorporating 0.25 mol% of the fluorescently-labeled lipid Lissamine rhodamine-PE in the lipid vesicles.

The final plasmid lipid ratio was 0.022, which corresponds to 88% entrapment. The resulting lipid-encapsulated therapeutic agent particles had an average size of 100 nm and a very small size distribution.

Using this invention, and the teachings of this specification, those skilled in the art will be able to identify other methods and means for generating fully encapsulated lipid-therapeutic agent particles, all of which are encompassed by the claims set out below.

Claims

1. A method for preparing fully lipid-encapsulated therapeutic agent particles of a charged therapeutic agent comprising the steps of

combining a lipid composition comprising preformed lipid vesicles in a first solvent with a therapeutic agent solution comprising the charged therapeutic agent in a second solvent to form a mixture, wherein said first and second solvent may be the same or different, provided that the two solvents are miscible and that the mixture has a resulting solvent composition which does not disrupt the preformed lipid vesicles; and

incubating the mixture for a period of time sufficient to allow the encapsulation of the therapeutic agent within the preformed lipid vesicles, wherein the preformed lipid vesicles comprise a charged lipid which has a charge which is opposite to the charge of the charged therapeutic agent.

2. The method of claim 1, wherein the preformed lipid vesicles have an average diameter of less than 200 nm.

3. The method of claim 3, wherein the preformed lipid vesicles have an average diameter of 80-150 nm.

4. The method of claim 3, wherein the preformed lipid vesicles have an average diameter of 90-100 nm.

5. The method of any of claims 1-4, wherein the therapeutic agent is an anionic compound and the charged lipid is cationic.

6. The method of claim 5, wherein the therapeutic agent is a polynucleotide.

7. The method of claim 5 or 6, wherein the cationic lipid is selected from the group consisting of

dioleyl-N,N-dimethylammonium chloride ("DODAC");

N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA");

N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP");

3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol");

N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE");

cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE");

cationic liposomes comprising N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA") and DOPE;

cationic lipids comprising dioctadecylamidoglycyl carboxyspermine ("DOGS") in ethanol;

N-(2,3-dioleyloxy)propyl)-N,N-dimethylammonium chloride ("DODMA") and

1,2-Dioleoyl-3-dimethylammonium-propane ("DODAP").

8. The method of any of claims 1-7, wherein the therapeutic agent solution is added to a reservoir containing the lipid composition.

9. The method of claim 8, wherein the reservoir has a volume of at 20 liters or greater, and contains at least 5 liters of lipid composition.

10. The method of claim 8 or 9, wherein the therapeutic agent solution is added to the reservoir through a dispensing head having one or more injection ports, each having a diameters of less than 2.0 mm.

11. The method of claim 10, wherein the therapeutic agent solution is introduced through a plurality of ports, each having a diameters of less than 1.0 mm.

12. The method of any of claims 1-10, further comprising an extrusion step in which the particles formed are passed through a sizing membrane having a pore size of less than 250 nm for at most ten extrusion cycles to produce a sized product containing lipid-encapsulated therapeutic agent particles of substantial uniform diameters less than 200.

13. The method of any of claims 1-12, wherein the lipid composition has a lipid concentration of 25 mM or less.

14. The method of any of claims 1-13, wherein the therapeutic agent is a polynucleotide and the therapeutic agent solution has a polynucleotide concentration of 2.0 mg/ml or less.

15. The method of any of claims 1-14, wherein the lipid composition comprises 10 to 40 mol % of the charged lipid, 25 to 40 mol % of a neutral lipid; 35 to 55 mol % of a sterol, and 0.5 to 15 mol % of a lipid having a steric barrier moiety for prevention of aggregation.

16. An apparatus for preparation of a lipid vesicles comprising:

(a) a first reservoir for receiving a buffer composition;

(b) a mixer for agitating buffer composition in the first reservoir;

(c) a second reservoir for receiving a lipid solution;

(d) a dispensing head for introducing lipid solution from the second reservoir into the first reservoir, and

(e) a connector joining the second reservoir to the dispensing head for conducting lipid solution from the second reservoir to the dispensing head, wherein the dispensing head has formed therein one or more injection ports having a diameter of 2 mm or less.

17. The apparatus of claim 16, wherein the mixer is a dynamic mixer.

18. The apparatus of claim 16, wherein the mixer is a static mixer.

19. The apparatus of any of claims 16-18, wherein the dispensing head has a plurality of injection ports formed therein.

20. The apparatus of claim 19, wherein the dispensing head has twenty or more injection ports formed therein.

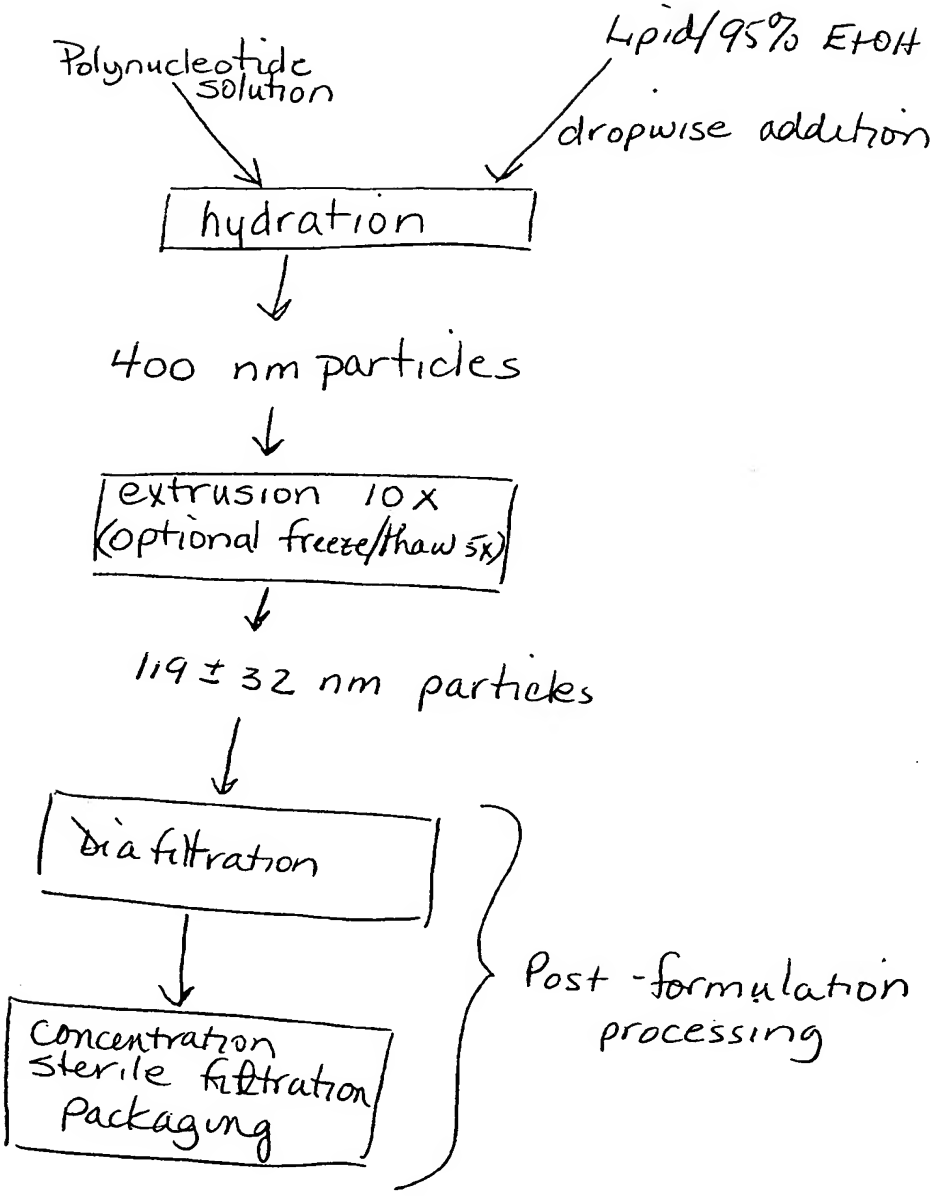

Fig 1 - prior art

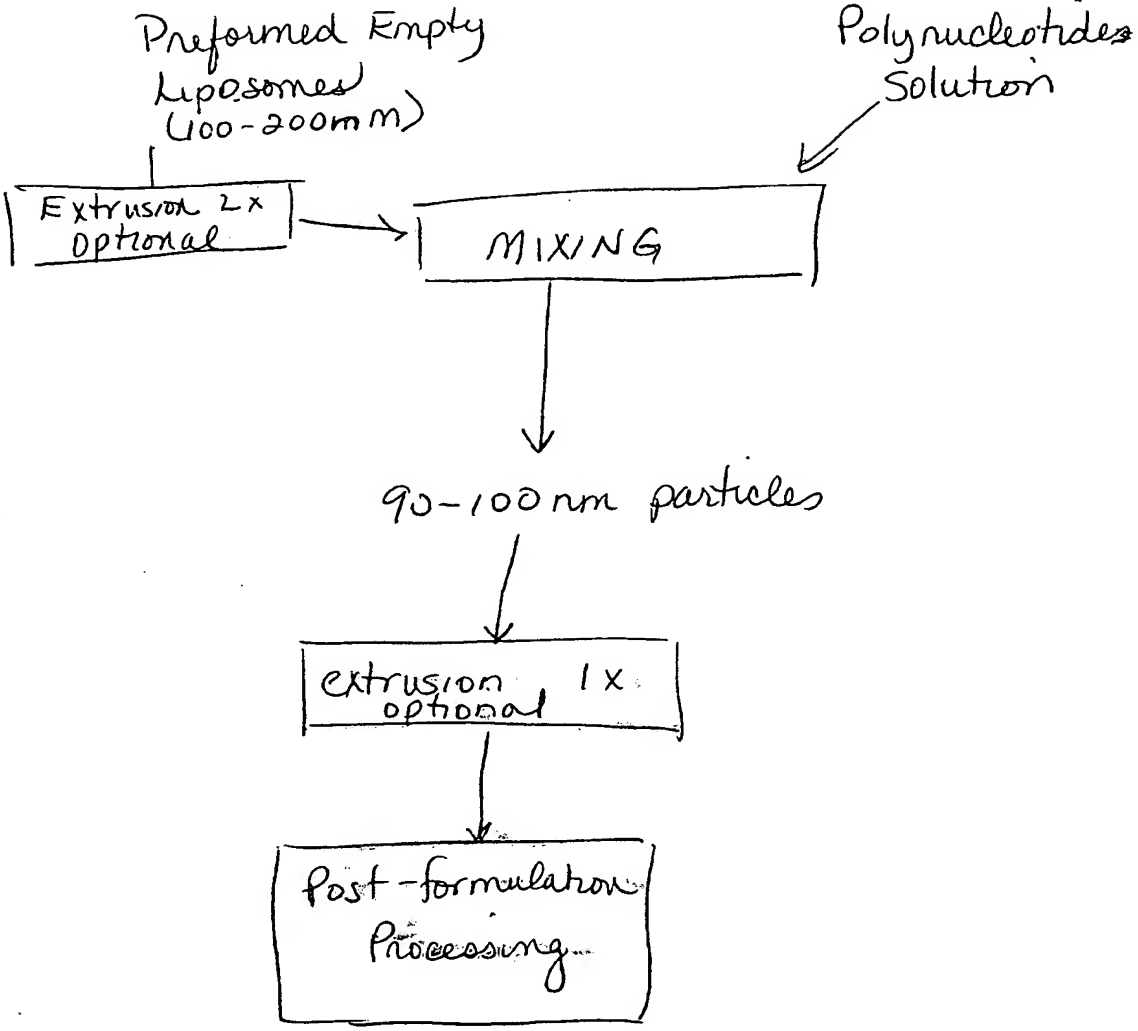
Preformed Empty Liposomes (100-200nm)
Polynucleotides Solution
Extrusion 2x optional
MIXING
90-100nm particles
extrusion 1x optional
Post-formulation Processing...

Fig 2

Fig 3

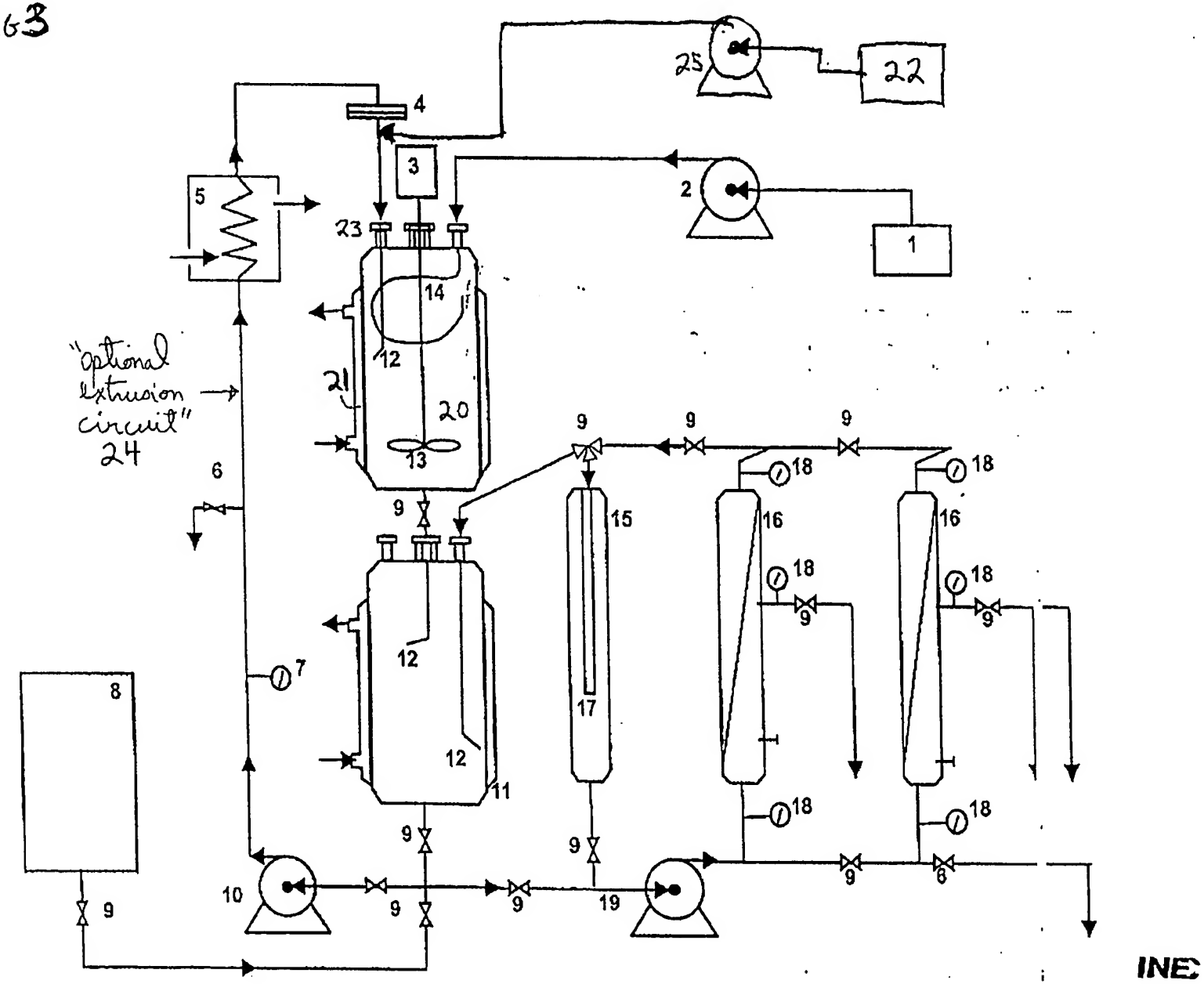

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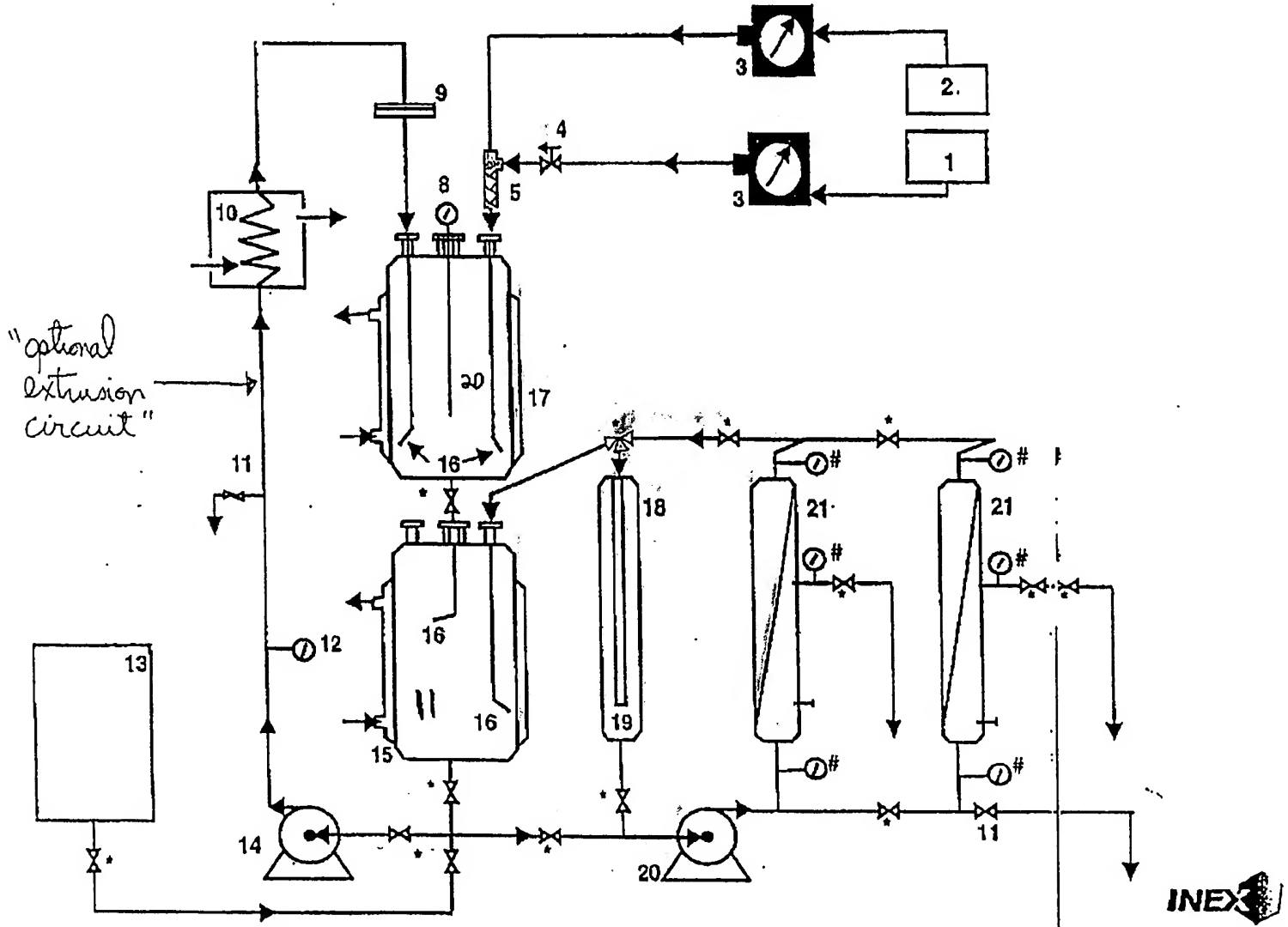
FIG 4



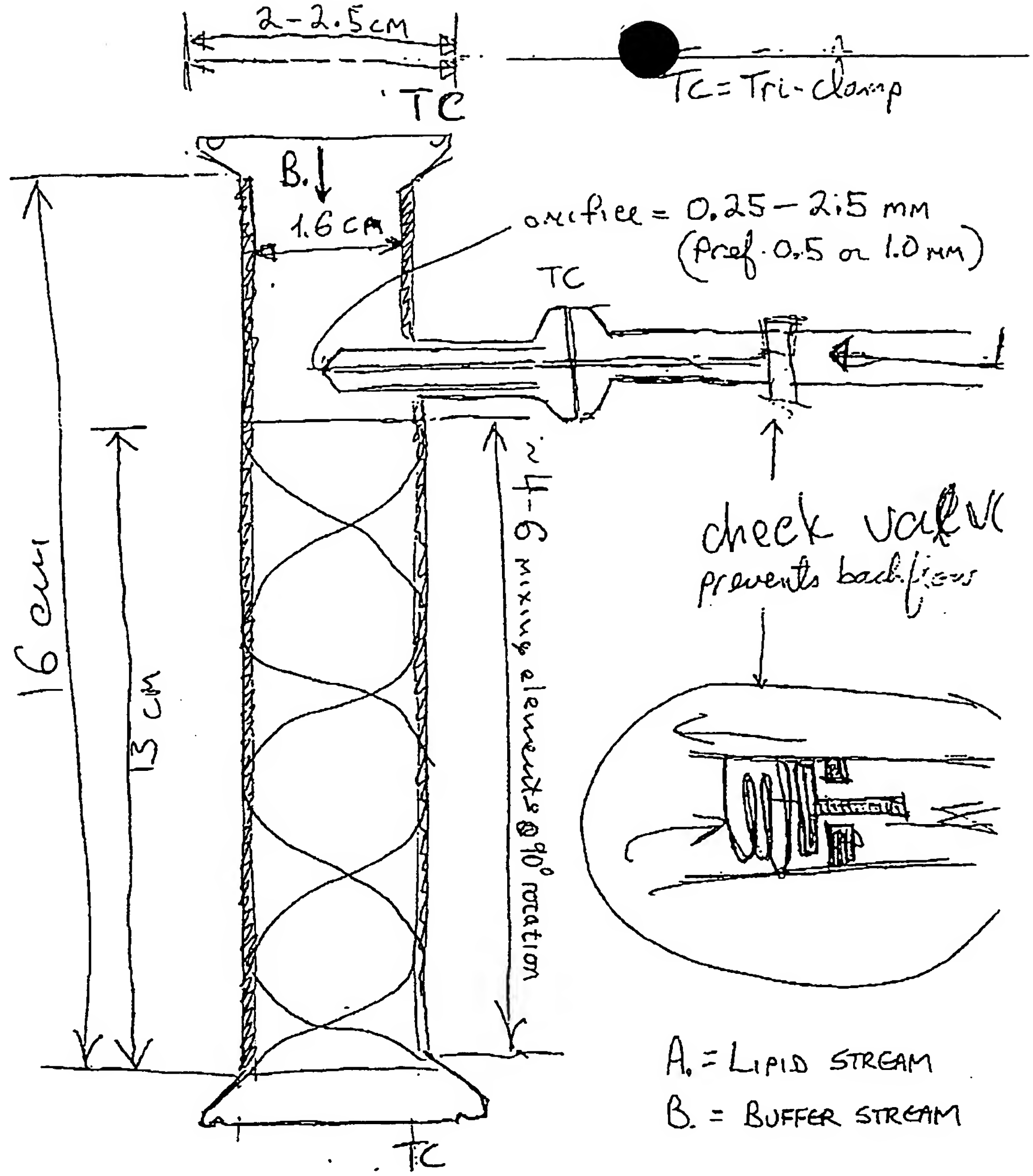
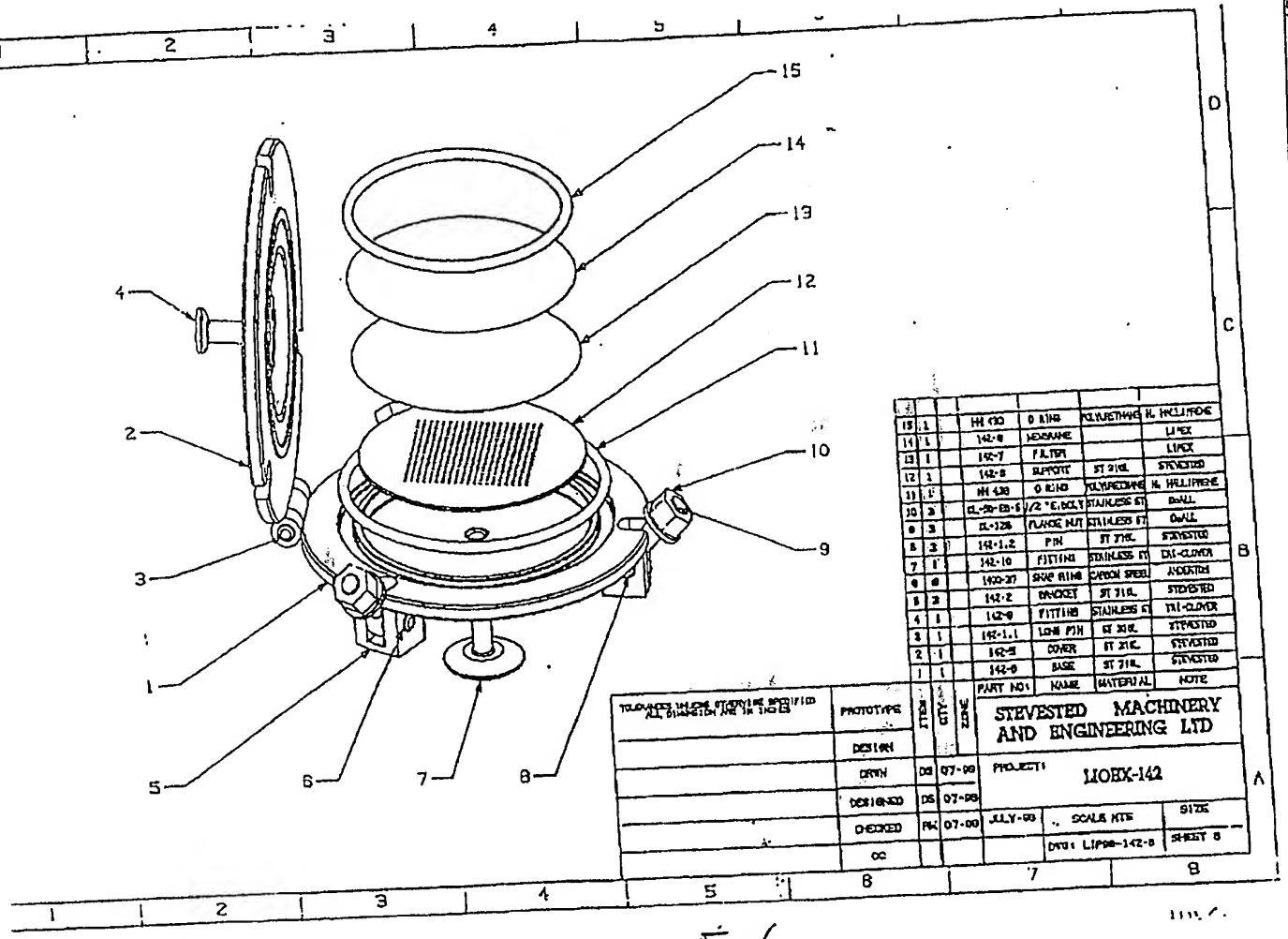


FIG. 5 STATIC MIXER FOR LIPOSOME PREPARATION

most important
1. Internal diameter
2. Length of mixing section



ITEM	QTY	PART NO:	NAME	MATERIAL	NOTE
15	1	HH 130	O RING	POLYURETHANE	H. HELLIPRENE
14	1	142-6	MEMBRANE		LIPEX
13	1	142-7	FILTER		LIPEX
12	1	142-5	SUPPORT	ST 316L	STEVESTED
11	1	HH 438	O RING	POLYURETHANE	H. HELLIPRENE
10	3	CL-50-ES-6	1/2 "E/BOLT	STAINLESS ST	DeALL
9	3	CL-126	FLANGE NUT	STAINLESS ST	DeALL
8	3	142-1.2	PIN	ST 316L	STEVESTED
7	1	142-10	FITTING	STAINLESS ST	TRI-CLOVER
6	6	1420-37	SNAP RING	CARBON STEEL	ANDERTON
5	3	142-2	BRACKET	ST 316L	STEVESTED
4	1	142-9	FITTING	STAINLESS ST	TRI-CLOVER
3	1	142-1.1	LONG PIN	ST 316L	STEVESTED
2	1	142-11	COVER	ST 316L	STEVESTED
1	1	142-0	BASE	ST 316L	STEVESTED

TOLERANCES UNLESS OTHERWISE SPECIFIED ALL DIMENSIONS ARE IN INCHES

PROTOTYPE			
DESIGN			
DRWN	DS	07-99	
DESIGNED	DS	07-99	
CHECKED	PK	07-00	
QC			

STEVESTED MACHINERY AND ENGINEERING LTD

PROJECT: LIQHX-142

JULY-99 | SCALE NTS | SIZE

DWG: LIPSB-142-0 | SHEET 8

Fig 6